Interleukin-2 Stimulates Resting Human T Lymphocytes' Response to Allogeneic, Gamma Interferon–Treated Keratinocytes*

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In order to investigate the biologic significance of HLA-DR expression by human keratinocytes, we have examined the possibility that DR-positive keratinocytes become alloantigen presenting cells for resting T cells in the presence of interleukin-2. Using this system, gamma interferon–treated, DR-positive keratinocytes stimulate the proliferation of allogeneic, resting T cells approximately 3-fold whereas non-gamma interferon–treated, DR-negative keratinocytes do not. Because a monoclonal antibody against recombinant gamma interferon inhibits this proliferation, the stimulation is dependent on pre-incubation with gamma interferon. By contrast, since the stimulation is not inhibited by a monoclonal antibody against HLA-DR, it is not clear that the stimulation is due to class II antigen expression by keratinocytes. To rule out that gamma interferon increases the expression of class I antigens, leading to stimulation of resting T cells on that basis, we determined whether gamma interferon treatment enhances class I antigen expression by keratinocytes. The lymphokine treated cells did not demonstrate more class I antigen expression than untreated keratinocytes. Thus, the observed stimulation of allogeneic, resting T cells by gamma interferon–treated keratinocytes in the presence of IL-2 is not due to increased class I antigen expression but is due to other cell surface antigen(s) induced by recombinant gamma interferon treatment. These results suggest that gamma interferon–exposed keratinocytes in the presence of interleukin-2 may augment the activation of resting T lymphocytes and, in this manner, may contribute to cutaneous inflammation. *J Invest Dermatol 89:464–468, 1987*

Recent studies have shown that human recombinant gamma interferon (rIFN-gamma) induces the synthesis and expression of HLA-DR antigen, a class II antigen of the major histocompatibility complex (MHC), in cultured, human keratinocytes [1,2]. This antigen is necessary for antigen presentation by macrophage to circulating T lymphocytes [3]. In order to determine the biologic consequences of HLA-DR expression by keratinocytes, we investigated the ability of these cells to stimulate allogeneic peripheral blood mononuclear leukocytes (PBML) in vitro and found these interactions to be complex [4]. For example, cultured keratinocytes produce prostaglandin E_2 (PGE_2), which inhibits PBML proliferation, and rIFN-gamma greatly increases PGE2 production [4]. Moreover, our results suggested that HLA-DR-positive ($HLA\text{-}DR^+$), allogeneic keratinocytes when incubated with PBML caused gamma interferon secretion which can represent a sign of PBML activation. These PBML did not proliferate markedly, however, as documented by low levels of [^{3}H]thymidine ([^{3}H]Thd) incorporation as well as by no increase in numbers of PBML [4].

Umetsu and associates have reported recently that $HLA\text{-}DR^+$ fibroblasts but not HLA-DR-negative ($HLA\text{-}DR^-$) fibroblasts can present tetanus toxoid to T cell clones but not to resting T (T_R) cells [5]. Furthermore, DR^+ fibroblasts had the capacity to stimulate allogeneic T_R cells but only in the presence of interleukin-2 (IL-2) [5]. Since T_R cell proliferation requires two sequential signals: first, "activation" as a response to foreign antigen, and secondly, a proliferative signal provided by IL-2 [6], we inves-

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Abbreviations:
- AZ: sodium azide
- EDTA: ethylenediamine tetraacetate
- FACS: fluorescence-activated cell sorter
- FCS: fetal calf serum
- GVHD: graft versus host disease
- $HLA\text{-}DR^-$: HLA-DR negative
- $HLA\text{-}DR^+$: HLA-DR positive
- [^{3}H]Thd: tritiated thymidine
- IL-1: interleukin-1
- KLR: keratinocyte lymphocyte reaction
- KTGF: keratinocyte-derived T-cell growth factor
- mAb: monoclonal antibody
- MHC: major histocompatibility complex
- PBML: peripheral blood mononuclear leukocytes
- PBS: phosphate-buffered saline
- PGE 2: prostaglandin-E2
- RBC: red blood cells
- rIFN-gamma: recombinant gamma interferon
- rIL-2: recombinant interleukin-2
- R/M-FITC: fluorescein isothiocyanate conjugated rabbit antimouse IgG
- SRBC: sheep red blood cells
- T_R: resting T cells

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tigated the effects of recombinant IL-2 (rIL-2) on the capacity of cultured, HLA-DR$^+$ keratinocytes to stimulate allogeneic T_R cells.

MATERIALS AND METHODS

Lymphokines and Monoclonal Antibodies (mAb) Ultrapure interleukin-1 (IL-1) (specific activity 8×10^6 U/μg protein) was obtained from Genzyme (Boston, Massachusetts), and a highly purified preparation of rIL-2 was obtained from Ed Engleman, Stanford, California. The mAb L243 was obtained from Becton Dickinson, Mountain View, California and also as tissue culture supernatant (azide free) from R. Levy, Stanford, California. The mAbs 17F12 (which stains all T cells) and CA1.41 (which labels HLA-DR) were obtained from Ed Engleman [7,8]. The rIFN-gamma and mAb against rIFN-gamma were a gift of M. Shepard, Genentech, Inc., South San Francisco, California, and mAb PA 2.6 against class I antigen was contributed by Peter Parham, Stanford, California [9].

Immunofluorescence Staining and FACS Analysis One million epidermal cells were stained for 30 min with a murine mAb diluted in 5% heat-inactivated, fetal calf serum (FCS) in phosphate-buffered saline (PBS) containing 0.02% sodium azide (AZ) as described previously [10]. The cells then were washed with 5% FCS/PBS/AZ, stained with fluorescein isothiocyanate–conjugated rabbit antimouse IgG (R/M-FITC) (ICN Immunobiologicals, Lisle, IL.) for 30 min, washed with and then resuspended in 5% FCS/PBS/AZ. The number of fluorescent cells was determined by fluorescence microscopy or FACS analysis [10].

Isolation of Resting T Cells Peripheral blood mononuclear leukocytes were collected by Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, New Jersey) separation of defibrinated blood as described [11]. The cells were washed with PBS and resuspended at 5×10^6 cells/ml in 40% absorbed FCS in PBS. Fresh sheep red blood cells (SRBC) were washed 3 times with PBS and adjusted to 3% v/v in 40% absorbed (on SRBC) FCS/PBS. Equal volumes of 3% SRBC and PBML were mixed gently. Ten to twenty milliliters of this cell suspension was layered on 10 ml Ficoll-Paque, incubated on ice for 30 min, and centrifuged at 2000 rpm at 4°C for 30 min. The interface, supernatant, and ficoll layers were removed by suction. To remove the SRBC the pellet was lysed with 10 ml ACK buffer (0.874% NH_4Cl, 0.1% $KHCO_3$, 0.0037% EDTA at pH 7.4), vortexed and incubated for 5 min at room temperature [8]. Ten ml of RPMI 1640 (Gibco, Grand Island, New York) plus 10% heat-inactivated, pooled human serum and 2 mM L-glutamine (complete RPMI medium) was added, the T cells were spun out and resuspended in 10 ml complete RPMI medium.

To remove activated, DR$^+$ cells, the isolated T cells were washed once with PBS, incubated on ice for 1 h in an antibody mixture (anti-Leu 10, anti-HLA-DR and Ca 1.41 [a mAb against HLA-DR]) and the DR$^+$ T cells and Leu 10 positive cells were lysed with complement (Low-Tox-H rabbit complement, Cedarlane Labs Ltd., Hornby, Ontario, Canada) for 45 min at 37°C [12]. Five milliliters of complete RPMI was added; the remaining cells (T_R cells) were spun, resuspended and counted.

Keratinocyte-Lymphocyte Reaction (KLR) Single cell suspensions of normal skin obtained at the time of face lift surgery were prepared according to the method of Liu and Karasek [13]. Small, round, viable cells were seeded on 3.5 cm collagen coated Petri dishes (Lux, Flow Laboratories, McLean, Virginia) or 96-well flat bottom microtiter plates (Linbro, Flow Lab.) in Dulbecco's modified Eagle's medium (DMEM) containing 1.4 mM calcium, supplemented with 10% heat-inactivated FCS, 50 μg/ml of gentamicin, and 2 mM L-glutamine (complete medium) as described previously [4]. Routinely, $2.5–5.0 \times 10^4$ viable cells were seeded per flat bottom microtiter well. Viability as determined by trypan blue exclusion immediately following trypsinization was 90% or better. The cells were maintained in a humidified incubator with 5% CO_2:95% air at 37°. The rIFN-gamma (100 U/ml) and indomethacin (Sigma Chemical Co., St. Louis, Missouri) (1 μg/ml) were added 2 to 3 days after cell seeding. Just before addition of allogeneic T_R cells (10^5 cells/well), the keratinocyte cultures were irradiated with 2,000 rads using a Cesium source, washed twice to remove rIFN-gamma, and the KLR was set up in complete RPMI medium plus indocin (1 μg/ml). The keratinocytes were irradiated to prevent proliferation of these cells in the microtiter wells. Interleukin-2 (5U/ml) was added right after the addition of the T_R cells. The KLR was set up in complete RPMI medium plus indocin (1 μg/ml). After 6 days of incubation, 10 μl of 0.1 mCi/ml ^{3}[H]Thd (sp act 6 Ci/mmol; (ICN, Irvine, California)) were added per well and the cultures harvested 18 h later on a PHD cell harvester (Cambridge Technology, Cambridge, MA) [4].

RESULTS

HLA-DR$^+$ Keratinocytes Do Not Stimulate Allogeneic Resting T Cells After HLA-DR antigen expression was induced on keratinocytes grown on collagen-coated microtiter wells by incubation with rIFN-gamma (100 U/ml), the cells were washed carefully and allogeneic T_R cells were added (10 cells^5/well). After 7 days of incubation, DR$^+$ keratinocytes alone did not stimulate proliferation of T_R cells as monitored by ^{3}[H]Thd incorporation (Table I).

Effects of Exogenous IL-1 and IL-2 on the KLR Using DR$^+$ Keratinocytes Since one explanation for this lack of stimulation might be that the DR$^+$ cells in the KLR are not able to provide enough IL-1 and/or IL-2 to support proliferation of T_R cells or that the keratinocytes might produce an inhibitor of these 2 cytokines, we added exogenous IL-1 and IL-2 to the KLR. Addition of 1 U/ml IL-1 did not cause the DR$^+$ keratinocytes to stimulate allogeneic T_R cells (Table I). By contrast, addition of exogenous IL-2 (5 U/ml) resulted in an approximately 3-fold stimulation of allogeneic T_R cells by DR$^+$ compared to DR$^-$ keratinocytes (Table I). This type of experiment, using 2 different responder T_R cells each time, showed the same trend on 5 separate occasions.

Table I. Stimulation of Allogeneic T_R Cells by DR$^+$ Keratinocytes in the Presence of IL-2

Keratinocyte Phenotype	Addition	Response of Allogeneic T_R cells (cpm)	
		Donor A	Donor B
DR$^-$	none	416 ± 13	351 ± 85
	IL-1[a]	1,512 ± 1,762	606 ± 159
	IL-2[b]	3,743 ± 1,247	1,570 ± 348
	IL-1 + IL-2	3,151 ± 150	1,407 ± 270
DR$^+$	none	275 ± 144	343 ± 156
	IL-1	342 ± 112	457 ± 247
	IL-2	11,238 ± 652	4,966 ± 1,515
	IL-1 + IL-2	11,474 ± 3,501	4,581 ± 1,386

[a] 1 U/ml IL-1
[b] 5 U/ml IL-2

Table II. Effect of Preincubation With a Monoclonal Antibody Against Gamma Interferon on the Capacity of Gamma Interferon–Treated Keratinocytes to Stimulate Allogeneic T_R Cells[a]

Addition 1	Addition 2	Response of Allogeneic T_R Cells (cpm) Donor A	Donor B
none	none	1,132 ± 74	5,485 ± 780
	mAb against rIFN-gamma	1,181 ± 78	4,858 ± 1,252
rIFN-gamma	none	6,548 ± 2,003	13,417 ± 2,201
	mAb against rIFN-gamma	1,979 ± 639	5,301 ± 1,893

[a]All of the microtiter wells received 5 U/ml IL-2.

The addition of IL-1 plus IL-2 did not increase the levels of incorporation of [^{3}H]Thd above those seen with IL-2 alone (Table I). Thus, enough IL-1 appears to be present in the KLR to activate the T_R cells maximally.

Effect of the Addition of mAbs Against rIFN-gamma and HLA-DR on the KLR To determine whether a mAb against rIFN-gamma would inhibit the KLR (using DR$^+$ keratinocytes plus IL-2), we added this mAb (300 neutralizing U/ml) to the keratinocytes in a separate series of microtiter plates at the same time as the rIFN-gamma (100 U/ml) was added. At the end of the 4 day incubation, we harvested representative microtiter wells and stained the keratinocytes with mAb against HLA-DR and determined cell fluorescence using fluorescence microscopy. Wells that had received only rIFN-gamma showed bright fluorescence on about 60% of the cells. The keratinocytes that had received the rIFN-gamma plus the mAb against this lymphokine showed only a few weakly fluorescent cells (less than 5%). Thus, the mAb against rIFN-gamma almost totally inhibited HLA-DR expression by keratinocytes. To representative microtiter wells seeded in parallel in this experiment, we added allogeneic T_R cells plus IL-2 and monitored incorporation of [^{3}H]Thd 7 days later. The concomittant addition of a mAb against rIFN-gamma inhibited the stimulation of allogeneic T_R cells by rIFN-gamma treated cells (Table II). Thus, a cell surface antigen induced by rIFN-gamma appears to be responsible for the observed stimulation by DR$^+$ keratinocytes.

To determine whether a mAb against HLA-DR (L243) would inhibit the stimulation of allogeneic T_R cells by DR$^+$ keratinocytes, we incubated the keratinocytes with L243 (1:50 dilution of tissue culture supernatant) in the microtiter wells during the entire 7 day incubation with T_R cells (Table III). Using 2 different responders, and repeated on 2 separate occasions, L243 did not inhibit the stimulation of allogeneic resting T_R cells by DR$^+$ keratinocytes in the presence of exogenous IL-2; however, in neither of these experiments did the mAb inhibit the mixed lymphocyte reaction (MLR) performed in parallel. On other occasions, however, we did observe about 50% inhibition of the MLR by mAb L243 at the 1:50 dilution. In a separate experiment using immunofluorescence, we also determined whether L243 would bind to DR$^+$ keratinocytes at the dilution of the supernatant used. We found that supernatant L243 diluted 1:50 binds to the HLA-DR antigen expressed by keratinocytes after incubation with rIFN-gamma.

Effect of rIFN-gamma on Expression of Class I Antigens of the MHC It has been reported that gamma IFN stimulates the expression of class I antigens in some murine tissues [14]. Therefore, we examined whether rIFN-gamma enhances class I antigen expression by human keratinocytes. We incubated keratinocytes with 100 U/ml rIFN-gamma for 4 days and determined class I antigen expression at the end of this time using staining with mAb PA 2.6 and FACS analysis. No stimulation of class I antigen occurred (not shown). In a parallel series of cultures, done as controls, the rIFN-gamma was capable of induction of class II antigen expression by these cells.

DISCUSSION

HLA-DR$^+$ but not HLA-DR$^-$ keratinocytes stimulate the proliferation of allogeneic T_R cells in the presence of exogenous IL-2. Without the addition of rIL-2, DR$^+$ keratinocytes do not stimulate allogeneic T_R cells. This result is in agreement with a recent report using a murine model [15]. In the presence of IL-2, however, DR$^+$ keratinocytes can stimulate T_R cells to proliferate. Since no macrophage or other antigen-presenting cells are present in the stimulating cell population in our system, DR$^+$ keratinocytes appear to act as alloantigen-presenting cells. Preincubation of the keratinocytes with a mAb against rIFN-gamma in the presence of rIFN-gamma greatly reduces induction of HLA-DR antigen expression by keratinocytes and concomitantly in-

Table III. Effect of a Monoclonal Antibody Against HLA-DR on Response of Allogeneic T_R Cells to DR$^+$ Keratinocytes

Keratinocyte Phenotype[a]	Addition	Response of Allogeneic T_R Cells (cpm) Donor A	Donor B
DR$^-$	none	2,481 ± 821	1,492 ± 187
	17F12[b]	3,355 ± 1,605	1,584 ± 313
	L243	2,063 ± 463	1,378 ± 211
DR$^+$	none	6,485 ± 1,536[c]	3,231 ± 983[c]
	17F12	10,087 ± 5,957	5,377 ± 755
	L243	3,956 ± 1,035[d]	3,094 ± 1,777[d]
		Response of PBML to Allogeneic irradiated PBML (cpm)	
	Addition[a]	Donor A	Donor B
	none	24,124 ± 6,990	30,869 ± 3,727
	17F12[b]	33,023 ± 5,578	45,087 ± 3,837
	L243	18,744 ± 2,496	26,807 ± 1,611

[a]All cultures contained 5 U/ml IL-2.
[b]This mAb has the same isotype as L243 and serves as a control.
[c]Statistically significant stimulation ($p < 0.05$).
[d]No statistically significant inhibition ($p > 0.05$).

hibits the capacity of these keratinocytes to act as alloantigen-presenting cells. Thus, the proliferative response of T_R cells to rIFN-gamma–treated keratinocytes is mediated by an effect of rIFN-gamma, not an impurity in the rIFN-gamma preparation. Moreover, it appears likely that the stimulation is due to expression of HLA-DR since expression of this antigen is correlated to the keratinocytes' capacity to stimulate the allogeneic T_R cells. Since a mAb against HLA-DR did not abrogate the capacity of DR^+ keratinocytes to stimulate allogeneic T_R cells, we cannot say with certainty that the stimulation is due to DR expression. In this connection, in our hands, the mAb L243 inhibited the classic mixed lymphocyte culture by maximally 50% and in several experiments no inhibition at all occurred. Thus, from our data it is unclear whether the inability of mAb L243 to inhibit the stimulation of allogeneic T_R cells by DR^+ keratinocytes is due to vagaries of the mAbs against HLA-DR [16,17] or that the observed stimulation is due to an antigen other than DR.

Other class II antigens, such as HLA-DR and HLA-DQ, have been described [18–20]. It is conceivable that these antigens are also induced by rIFN-gamma and that they are responsible for the observed stimulation. Experiments are currently in progress to determine whether DQ and DP expression can be induced on keratinocytes by rIFN-gamma.

The $OKT8^+$ subpopulation of peripheral blood T cells can recognize class I antigens and can respond to these antigens by proliferation in the presence of lymphokines such as IL-2 [17]. Since it has been reported that rIFN-gamma induces increased expression of class I antigen of the MHC in certain murine tissues [14], we determined whether an increased expression of class I antigen might explain the observed stimulation of resting T cells by rIFN-gamma treated keratinocytes. Our data do not indicate that rIFN-gamma induces increased expression of this antigen on these cells. On the other hand, we can not rule out the possibility that another cell surface antigen is induced by rIFN-gamma and that this antigen either alone or in conjunction with a class II antigen is responsible for stimulation of the allogeneic T_R cells. For example, a non–H-2 alloantigen (Epa-1) that may represent a histocompatibility antigen has been described in mice [21]. In this regard, one of us has recently observed that treatment of keratinocytes with rIFN-gamma strongly enhances the binding of allogeneic PBML to the keratinocytes [22]. Thus, in addition to HLA-DR, rIFN-gamma may induce other cell surface antigens important in cell-cell recognition.

The reason for the necessity to add exogenous IL-2 in order to obtain stimulation in the KLR using DR^+ cells is unclear. Using a murine system, it has been demonstrated that epidermal cells secrete an IL-2 like factor (KTGF) [23]. Possibly, in our system using human cells, not enough IL-2 is generated to drive the reaction. Alternatively, using DR^+ cells a small amount of an inhibitor of IL-2 is generated and this can be overcome by the addition of excess IL-2. Another possibility is that, in this regard, the murine skin is different from the human. Other differences between the murine and human epidermis have recently been documented. For example, human epidermis does not appear to contain a $Thy\text{-}1^+$ cell [24], and murine rIFN-gamma does not cause Ia expression on murine keratinocytes (G. Kreuger, personal communication).

Schuler and coworkers have reported that T_R cells require a dendritic cell (i.e., Langerhans cell) to initiate proliferation [25]. Thus, cell surface expression of HLA-DR by a non–bone marrow derived cell (i.e. keratinocyte, fibroblast, endothelial cell) is not sufficient in itself to provide all the complex proliferative signals to T_R cells. Our results suggest, however, that if a source of IL-2 is present, DR^+ keratinocytes may play a role in the afferent limb of the immune response.

The experimental results documented here may explain why DR^+ keratinocytes occur in contact dermatitis and herpex simplex infection [26]. Conceivably, induction of DR expression by keratinocytes is a mechanism by which the body increases the number of antigen-presenting cells in a localized area. In this view, these DR^+ keratinocytes (plus foreign antigen and host IL-2) would be capable of stimulating proliferation of host lymphocytes. In some cases, this interaction of DR^+ keratinocytes with lymphocytes may not be turned off by the host and could then result in such lymphocyte-mediated skin diseases as lichen planus and lupus erythematosus [27]. Another possible mechanism for the etiology of lichen planus as well as autoimmune diseases is that the expression of DR in conjunction with other cell surface antigens native to the surface of this normally DR^- cell can initiate proliferative signals in autologous lymphocytes [28]. Finally, the first step in chronic graft versus host disease (GVHD) may be activation and proliferation of circulating, host T helper cells [29]. The production of alloreactive, cytotoxic T cells may represent a second (later) step in the process. This may explain why the presence of cytotoxic T cells alone is not sufficient to induce GVHD in animal models [30]. Experiments are currently in progress in this laboratory to further clarify these issues.

We previously have reported data which suggested that cultured keratinocytes secrete a substance which inhibits PBML proliferation. The current experiments do not further clarify this observation. The stimulation of T_R cells observed in most of the experiments, however, appeared to relate inversely to the number of keratinocytes seeded (not shown). Thus, in one of the later experiments in which we had seeded 2 different concentrations of keratinocytes in two separate microtiter plates, we saw stimulation only in the plate seeded with 2.5×10^4 keratinocytes/well but saw no stimulation in the plate seeded with 5×10^4 cells/well in which the media had turned very acid by the time the T_R cells were harvested. By contrast, stimulation of T_R cells increased when more T_R cells were added to the microtiter wells. Although the number of viable keratinocytes seeded can be measured, the number of cells that attach and their rate of growth cannot be predicted accurately. Therefore, we seeded microtiter plates with 2 concentrations of keratinocytes for the later experiments and then chose the plate which showed preconfluent keratinocytes to perform the assay using T_R cells.

The lack of stimulation of allogeneic T_R cells by keratinocytes (DR^+ or DR^-) in the absence of exogenous IL-2 may explain the lack of rejection of cultured, allogeneic epidermal cell sheets recently reported by Thivolet and colleagues [31]. These cultured epidermal cells presumably deprived of Langerhans cells by culture in vitro [32,33] would not provide a source of class II antigen and therefore may avoid detection by circulating host immune competent cells, either because of their anatomic location in the upper epidermis or because DR^+ cells are needed to attract circulating lymphocytes to the epidermis [34].

Finally, these results underscore the complexity of immune reactions in the skin. The keratinocyte can no longer be viewed as merely providing a barrier to separate the external and internal milieu. Rather, it seems likely that the keratinocyte, after exposure to gamma interferon, may play a role in initiating further interactions with the immune system.

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